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Applicant	: Rene Gantier et al.	Art Unit	: 1647
Serial No.	: 10/658,834	Examiner	: Stoica, Elly Gerald
Filed	: September 8, 2003	Conf. No.	: 7681
Cust. No.	: 20985		
Title	: **RATIONAL EVOLUTION OF CYTOKINES FOR HIGHER STABILITY, THE CYTOKINES AND ENCODING NUCLEIC ACID MOLECULES**		

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Manuel Vega, declare as follows:

1) I am a joint inventor of the above-captioned application. I am founder of Nautilus Biotech, which is the Applicant in the above-described application. I am currently Chief Executive Officer of Nautilus Biotech. I have held this position since January 2000. Prior to that time, I held senior management positions at Microgen SA from 1992 to 1996 and at Gènèthon from 1997-1999. I also was an adjunct Professor of Human Gene Therapy at the Universidad Nacional del Sur (UNS) from 1992-1997. I also have gained scientific research experience in Germany at Würzburg University, in France at the Institut National de la Santé et de la Recherche Médicale (INSERM), in the Netherlands at the Netherlands Organization for Applied Scientific Research (TNO) and in Argentina at Consejo Nacional de Investigaciones Cientificas y Técnicas (CONICET).

2) I received a Licentiate degree in Biological Sciences from the Universidad Nacional del Sur (UNS), Bahia Blanc, Argentina in 1982. I received a Doctor in Biology from UNS in 1987. I have over 20 years of experience in the field. I have authored or co-authored over 26 publications in scientific journals and book chapters, and I have received numerous awards for my scientific achievements.

3) I am familiar with the outstanding Office Action, and references cited therein.

4) The above-captioned application describes modified cytokines, including modified interferon alpha (IFN-alpha) cytokines, that are modified to be more resistant to proteases than cytokines that do not include such modifications. The application also describes

that protease resistant mutants can exhibit increased serum stability and half-life. In many instances, protease resistance can be achieved by virtue of only a single amino acid change. Protease resistance that is achieved by modification of as few as a single amino acid residues and/or fewer than all protease cleavage sites is manifested as increased resistance of the entire molecule to proteolysis in blood, tissue and the intestine. The polypeptides that exhibit increased resistance to proteases retain biological activity and can be administered at lower doses for subcutaneous administration compared to the unmodified protein. In addition, as described in the instant application, proteins exhibiting increased resistance to proteases can be successfully administered orally. Thus, proteins, particularly therapeutic proteins, containing only a few modifications in the primary sequence, particularly one or two modifications, can be formulated for oral administration without specific formulation requirements, other than the modified protein.

5) Using methods as described in the above-referenced application and standard methods as described herein, myself and other scientists involved in these projects have prepared modified cytokines that exhibit increased protease resistance. These include modified IFN-alpha cytokines that exhibit protease resistance by virtue of a single amino acid change in the primary sequence. It is the mutation in the primary sequence itself that renders the protein resistant to proteases, without any additional modifications. By changing only one or two amino acids in the protein, the modified IFN-alpha cytokines retain activity comparable to native levels. Such modified proteins are more resistant to proteases in the blood, serum and gastrointestinal tract resulting in increased pharmacokinetics as compared to IFN-alpha cytokines that do not have the modifications, including native IFN-alpha. These modified polypeptides can be administered by subcutaneous routes and also orally.

6) To demonstrate the properties of the modified cytokines, such as modified IFN-alpha cytokines described in the above-captioned application, a description of results obtained for exemplary modified cytokines follows. Data is provided for several modified IFN-alpha cytokines, including an IFN-alpha cytokine containing the mutation E41Q. The results show that it is possible to make a single amino acid change in the primary sequence and increase resistance to a variety of proteases and/or protease mixtures. In addition, the data show that the modified IFN-alpha cytokines, when administered orally or subcutaneously, exhibit increased pharmacokinetic profiles as compared to native IFN-alpha or the commercially available IFN-

alpha cytokine Intron A®. The increased pharmacokinetics means that modified IFN-alpha cytokines can be administered orally or subcutaneously, including less frequently or at lower doses, than current IFN-alpha therapeutics.

I. Methods

A. Resistance to Proteolysis

Modified IFN-alpha cytokines containing mutations were treated with proteases in order to identify resistant molecules. The relative resistance of the mutant proteins compared to the native protein against enzymatic cleavage was determined by exposure to a mixture of proteases (containing 1.5 pg of each of the following proteases (1% wt/wt, Sigma): α-chymotrypsin, carboxypeptidase, endoproteinase Arg-C, endoproteinase Asp-N, endoproteinase Glu-C, endoproteinase Lys-C, and trypsin) at 25°C for a set time period between 30 minutes to 120 minutes. At the end of the incubation time, 10 µl of anti-proteases complete medium containing mini EDTA free tablets, Roche (one tablet was dissolved in 10 ml of DMEM and then diluted to 1/1000) was added to each reaction in order to inhibit protease activity. Treated samples were then used to determine residual activity such as anti-viral or proliferative activity.

B. Anti-viral activity

Residual activity of modified IFN-alpha cytokines were assessed in an anti-viral assay. Anti-viral activity can be measured by cytopathic effects (CPE). Anti-viral activity of modified cytokines was determined by the capacity of the cytokine to protect HeLa cells against EMC (mouse encephalomyocarditis) virus-induced cytopathic effects. The day before, HeLa cells ($2x10^5$ cells/ml) were seeded in flat-bottomed 96-well plates containing 100 µl/well of Dulbecco's MEM-Glutamax-sodium pyruvate medium supplemented with 5% SVF and 0.2% of gentamicin. Cells were grown at 37°C in an atmosphere of 5% CO_2 for 24 hours.

Two-fold serial dilutions of modified cytokine samples preincubated with protease were made with MEM complete media into 96-deep well plates. Twenty-four (24) hours after seeding the cells, the medium was aspirated from each well and 100 µl of diluted samples were added to HeLa cells. Each sample dilution was assessed in triplicate. The last two rows of the plates were filled with 100 µl of medium without added sample in order to serve as controls for cells with and without virus. After 24 hours of growth, a 1/1000 EMC virus dilution solution was placed in each well, except for the cell control row. Plates were returned to the CO_2 incubator for 40 - 48 hours. The medium was discarded, and the cells were washed twice with 100 µl of 1X PBS and

stained for 1 hour with 80- 100 µl of staining solution (typan blue or ehanol-formamide-methyl blue mixture) to determine the proportion of intact cells. Plates were washed in a distilled water bath and the cell-bound dye was extracted using 80 - 100 µl of ethylene-glycol mono-ethyl-ether (Sigma). The absorbance of the dye was measured using an ELISA plate reader (Spectramax; Molecular devices) at 660 nm.

C. Subcutaneous administration in Cynomolgus Monkeys

Naïve cynomolgus monkeys (*Macaca fascicularis*; one male and one female for each subgroup) received subcutaneous (SC) injections of 0.3 mg/kg native IFN-alpha, Intron A® (a commercially available IFN-alpha), or selected modified IFN-alpha LEAD proteins, including a modified IFN-alpha containing an E41Q mutation. At various time points (pre-dose, 0.02 0.5, 1, 2, 4, 8, 12, 16, 24, 48, 72 h post-dose), blood was collected into anti-coagulant and anti-protease solution and the residual anti-viral activity was determined as described above.

D. Per-os (PO; oral route) administration to Cynomolgus Monkeys

A single dose (0.9 mg/kg) of enteric-coated capsule formulation of an E41Q mutant IFN-alpha and native IFN-alpha were tested after PO administration in Cynomolgus monkeys (*Macaca fascicularis*) in order to compare the pharmacokinetic and systemic profile of the two proteins after oral administration. A total of four purposely bred Cynomolgus monkeys, two males and two females, divided into two groups with one male and one female per group, were dosed with 0.9 mg/kg of an enteric-coated capsule formulation of modified IFN-alpha or native IFN-alpha by PO route. At various time points (day -1, 0, 0.5, 1, 1.5, 2, 4, 6, 8, 12, and 24 h) post-administration, a blood sample (1 ml) was taken from the saphenous or cephalic veins of all monkeys for the determination of the remaining anti-viral activity levels of modified IFN-alpha or native IFN-alpha in plasma. Each animal was checked for mortality and clinical signs at least twice a day during the treatment period. The body weight of each animal was recorded at least twice during the pre-treatment period.

II. Results

A. In Vitro Resistance to Proteolysis

Residual anti-viral activity was determined for exemplary IFN-alpha candidate LEAD cytokines and native IFN-alpha following treatment with protease mixture. As described in the application, 184 mutants of interferon-alpha (IFN-α2b), each containing a single amino acid change compared to native IFN-α2b, were generated and tested based on the predetermined

property of increased protease resistance. Candidate LEADs were tested *in vitro* for protease resistance by incubating 100 µl of 1500 pg/ml (500 U/ml) of IFNα-2b with a cocktail of proteases as described above. Following protease treatment, residual activity was assessed by an anti-viral assay. Table 1 shows the results of some of the tested modified IFN-alpha cytokines. In the Table, the resistance to proteolysis is indicated as "no change" or "increased" as compared to the residual activity of the respective native polypeptide under the same protease treatment conditions. The results show that many of the modified IFN-alpha cytokines tested exhibited an increased resistance to protease *in vitro* as compared to native IFN-alpha.

The data are not meant to be representative of all proteases, but are exemplary data showing the resistance to proteolysis to an exemplary protease cocktail as described in the methods above. Thus, the data are not comprehensive and are not meant to be indicative that other modified IFN-alpha cytokines do not exhibit protease resistance.

TABLE 1: Interferon Alpha LEADS

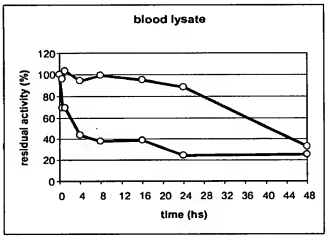
Mutant	Resistance to proteolysis
F27V	No change
R33H	No change
E41Q	Increased
E41H	No change
E58Q	Increased
E58H	Increased
E78Q	Increased
E78H	Increased
Y89H	No change
E107Q	Increased
E107H	Increased
P109A	No change
L110V	No change
M111V	No change
E113H	Increased
L117V	Increased
L117I	Increased
K121Q	Increased
R125H	Increased
R125Q	Increased
K133Q	Increased
E159H	Increased
E159Q	Increased

The E41Q IFN-alpha Lead protein was chosen for further kinetic analysis. The decreased susceptibility to proteases was evaluated by exposing E41Q mutant IFN-alpha and native IFN-

alpha to the following proteolytic treatments: a) human blood lysate; b) human serum; c) chymotrypsin (10% w/w); d) a protease mixture (1% w/w of α-chymotrypsin, carboxypeptidase, endoproteinase Arg-C, endoproteinase Asp-N, endoproteinase Glu-C, endoproteinase Lys-C and trypsin) as described above in the methods for assaying resistance to proteases, except that incubation with protease mixture was done for variable time. After the incubation, the protease reaction was stopped and the residual anti-viral activity was determined as described above. The results are shown below in Figure 1, A) – D). The results show that E41Q mutant IFN-alpha (blue line) maintained 50 – 90% of its antiviral activity for up to 24 hours, compared to native IFN-alpha (black line), which lost greater than 30% activity within 1 hour and maintained only 25-30% of its activity at 24 hours.

FIGURE 1:

A.



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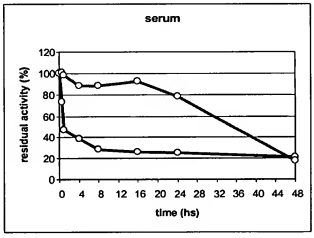

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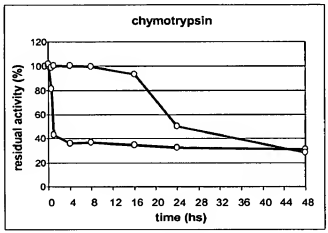

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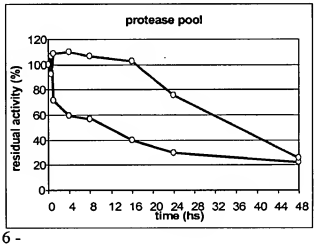


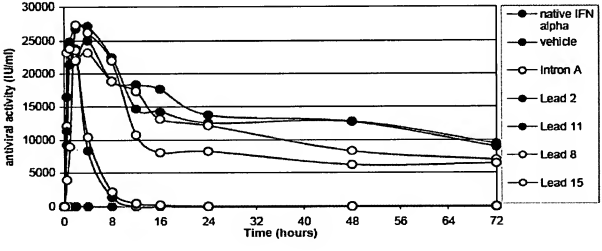
B. *In Vivo* Exposure

Selected modified IFN-alpha LEADs were assayed for their residual activity following exposure to proteases *in vivo* after subcutaneous or per-oral administration

1. Subcutaneous

Four modified IFN-alpha LEADS, each containing one or more single amino acid change described in the above-captioned application as compared to native IFN-α2b, were tested for their residual activity in plasma following subcutaneous administration. These included E41Q (Lead 11), as well as other Leads (Lead 2, 8 and 15) containing two to four amino acid replacements. The LEADs were assessed for their residual activity in serum following subcutaneous administration into Cynomolgus monkeys as compared to native IFN-alpha and an IFN-alpha sold commercially as Intron A®. After a single dose of 0.3 mg/kg of polypeptide, residual activity in the plasma was determined at various time-points post-administration. The results in Figure 2 show that both native IFN-alpha and Intron A® retained anti-viral activity that was barely detectable 8 hours after administration, with no detectable activity observed at later time points. All of the modified IFN-alpha LEADs tested retained anti-viral activity up to the last time point tested at 72 hours post-injection, with little decrease in anti-viral activity observed up to 8 hours post-injection of the modified IFN-alpha LEAD proteins. Based on these results, the half-life of native IFN-alpha is 1.5 hours and the half-life of E41Q mutant IFN-alpha is 44.9 hours. Thus, the E41Q mutant and other mutants tested have an improved pharmacokinetic profile and increased half-life compared to native IFN-alpha.

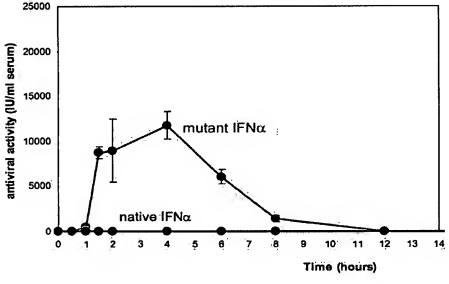
FIGURE 2



2. Oral Administration

The E41Q mutant IFN-alpha (mutant IFNα) LEAD containing a single amino acid change was investigated for its residual activity after per-os (PO; by oral route) administration. After a single dose of 0.9 mg/kg of enteric-coated capsule formulation of E41Q mutant IFN-alpha or native IFN-alpha by PO administration in Cynomolgus monkeys, residual anti-viral activity was determined in the plasma to compare the pharmacokinetic (PK) and the systemic exposure profile of the two proteins after oral administration. The results are depicted in Figure 3 below. The PK profiles of E41Q mutant IFN-alpha and native IFN-alpha detected in the blood circulation by anti-viral activity assay are represented as the average of the male and female monkeys for each molecule. The data show that following oral administration of E41Q mutant IFN-alpha in enteric-coated capsules a significant anti-viral activity was detected in monkey plasma, whereas no activity was detected in animals treated with native IFN-alpha at the same dose. The results evidence that a modified IFN-alpha cytokine, containing only a single mutation, exhibits a greatly improved resistance to protease compared to the native cytokine.

FIGURE 3



III. Conclusion

The data show that single or a few mutations can produce modified proteins that have improved pharmacological properties, such as increased half-life, and oral availability. Exemplified modified interferon-alpha cytokines containing only a single amino acid mutation in their primary sequence exhibit increased protease resistance compared to native interferon alpha. By virtue of the mutation, such proteins exhibit increased half-life as evidenced by increased anti-viral activity following subcutaneous administration. In addition, such IFN-alpha mutants also exhibited increased protease resistance to proteases in the gastrointestinal tract, and thereby exhibited activity in the bloodstream following oral administration. In contrast, the native IFN-alpha exhibited no activity following oral administration.

The results presented here demonstrate that protease-resistant IFN-alpha cytokines exhibit improved pharmacokinetic profiles compared to native IFN-alpha as evidenced by increased activity in the bloodstream following subcutaneous or per-oral administration. Thus, modified IFN-alpha cytokines made protease resistance by virtue of a single amino acid change can be administered orally or can be administered subcutaneously, including less frequently or at lower doses.

* * *

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

_____ _____

Date Manuel Vega